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Current Opinion in Biotechnology

Heading in the right direction: thermodynamics-based network analysis and pathway engineering

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Thermodynamics-based network analysis through the introduction of thermodynamic constraints in metabolic models allows a deeper analysis of metabolism and guides pathway engineering. The number and the areas of applications of thermodynamics-based network analysis methods have been increasing in the last ten years. We review recent applications of these methods and we identify the areas that such analysis can contribute significantly, and the needs for future developments. We find that organisms with multiple compartments and extremophiles present challenges for modeling and thermodynamics-based flux analysis. The evolution of current and new methods must also address the issues of the multiple alternatives in flux directionalities and the uncertainties and partial information from analytical methods.

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Current Opinion in Biotechnology 2015, **36**:176–182

This review comes from a themed issue on **Pathway engineering**

Edited by **Michael J Betenbaugh** and **William E Bentley**

For a complete overview see the Issue and the Editorial

Available online 16th September 2015

http://dx.doi.org/10.1016/j.copbio.2015.08.021

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Introduction

In the last 15 years, the number of annotated genome sequences has grown tremendously, and this has led to reconstruction of genome scale metabolic models (GEM) for many organisms, from unicellular prokaryotes to higher organisms such as mouse and human [1]. These metabolic models are *in silico* representations of all biochemical reactions that take place in the cell. Through various methods, such as Flux Balance Analysis (FBA), different phenotypes of organisms can be simulated and analyzed [2]. Directionalities and allowable flux ranges for metabolic reactions are the main constraints that delineate the boundaries for GEMs. The two most important uses of thermodynamics-based analysis of metabolic networks are the determination of reaction directionality and the estimation of how far from, or close to, equilibrium the reactions in the network operate.

In most of the metabolic flux balance studies that discuss and analyze reaction thermodynamics and energetics, the authors consider the reactions as irreversible (unidirectional) based on the standard Gibbs free energy of reaction. Soh and Hatzimanikatis [3,4] suggested differentiating between 'reaction directionality' and the commonly used term 'reaction reversibility'. Reaction reversibility is a *kinetic* property of the enzyme and it is used to denote that the enzymes are able to catalyze the reactions in both directions, that is, the *forward* and *backward* reactions. If an enzyme is catalytically reversible, then the directionality of the reaction depends on the displacement of the reaction from thermodynamic equilibrium. In the context of a metabolic network, with or without thermodynamic constraints, a reversible reaction can be either *bidirectional*, that is, it is able to operate in both the forward and reverse directions, or *unidirectional*, that is, it can operate only in one of the directions. The catalytic reversibility is an enzyme property that depends on the enzyme amino acid sequence, and therefore it can be different between organisms. The information about catalytic reversibility is available for a relatively small number of the enzymes in the biological databases and for a very small number of organisms. Therefore, by determining the reaction directionality, thermodynamic constraints provide important information that substitute for the lack of information about reaction reversibility.

Three main approaches have been used for the introduction of thermodynamic constraints (network thermodynamics): (i) the energy balance analysis (EBA) [5], (ii) the network-embedded thermodynamic analysis (NET analysis) [6,7], and (iii) the thermodynamics-based flux analysis (TFA), which has been also called thermodynamics-based metabolic flux analysis (TMFA) [8] or thermodynamics-based flux balance analysis (TFBA) [4]. All three methods introduce a new set of constraints that enforce the reactions fluxes to operate within the feasible bounds of energy constraints. The general EBA problems constrain the directionality and bounds of the fluxes using the value of the Gibbs free energy, either as a constant or as continuous variable within defined ranges. NET analysis and TFA constrain also the fluxes using the value of the Gibbs free energy but as a linear function of the logarithms of the metabolite concentrations (or activities). However, NET analysis requires a predetermination of the directionality of the fluxes, and the thermodynamic constraints determine if the flux is feasible in the defined direction and what are the

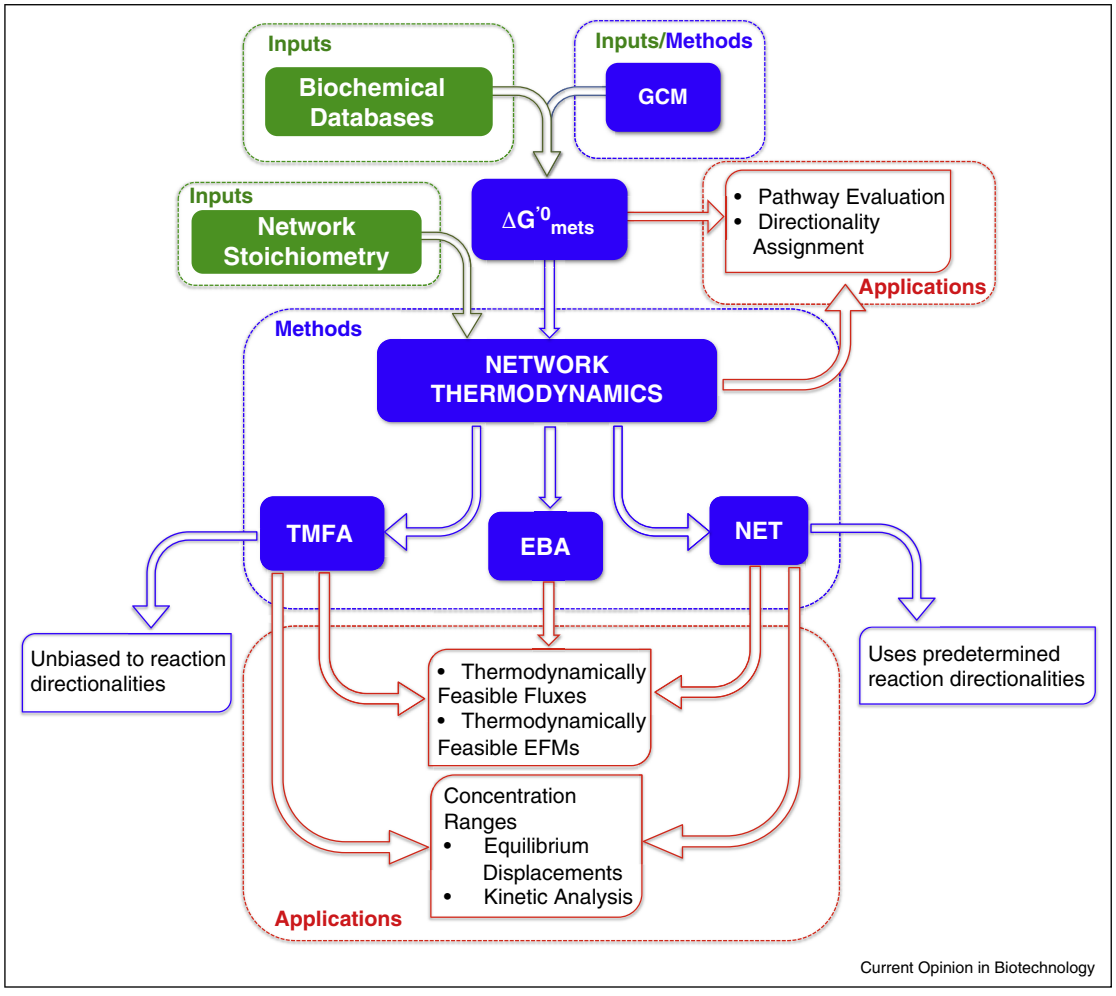
thermodynamically feasible concentration ranges. The TFA considers initially all catalytically reversible fluxes as thermodynamically bidirectional and employs a mixed-integer linear programming formulation that accounts for concentration ranges and it computes the flux directionality based on the thermodynamically feasible concentration profiles. Therefore TFA introduces the minimum bias about reaction directionality and it simultaneously computes thermodynamically feasible flux *and* concentration ranges. Moreover, the EBA and NET analysis formulations represent special cases of the TFA formulation. Hence, we believe that any analysis that uses thermodynamic constraints should apply TFA, or a similar formulation in order to avoid incomplete or false predictions about the properties of the network.

We review here the recent publications that have applied thermodynamic constraints in metabolic flux balance analysis. The major applications of thermodynamics have benefited the study of metabolism through three main uses: (i) the application of thermodynamic constraints to assign directionalities and thus constraint the allowable flux space and improve the predictions of metabolic modeling; (ii) the evaluation of the feasibility of synthetic and metabolically engineered pathways; and (iii) the integration of metabolomics data into metabolic models and their analysis and interpretation in the context of metabolic networks (Figure 1).

Assigning directionality based on Gibbs free energy of reactions in GEMs

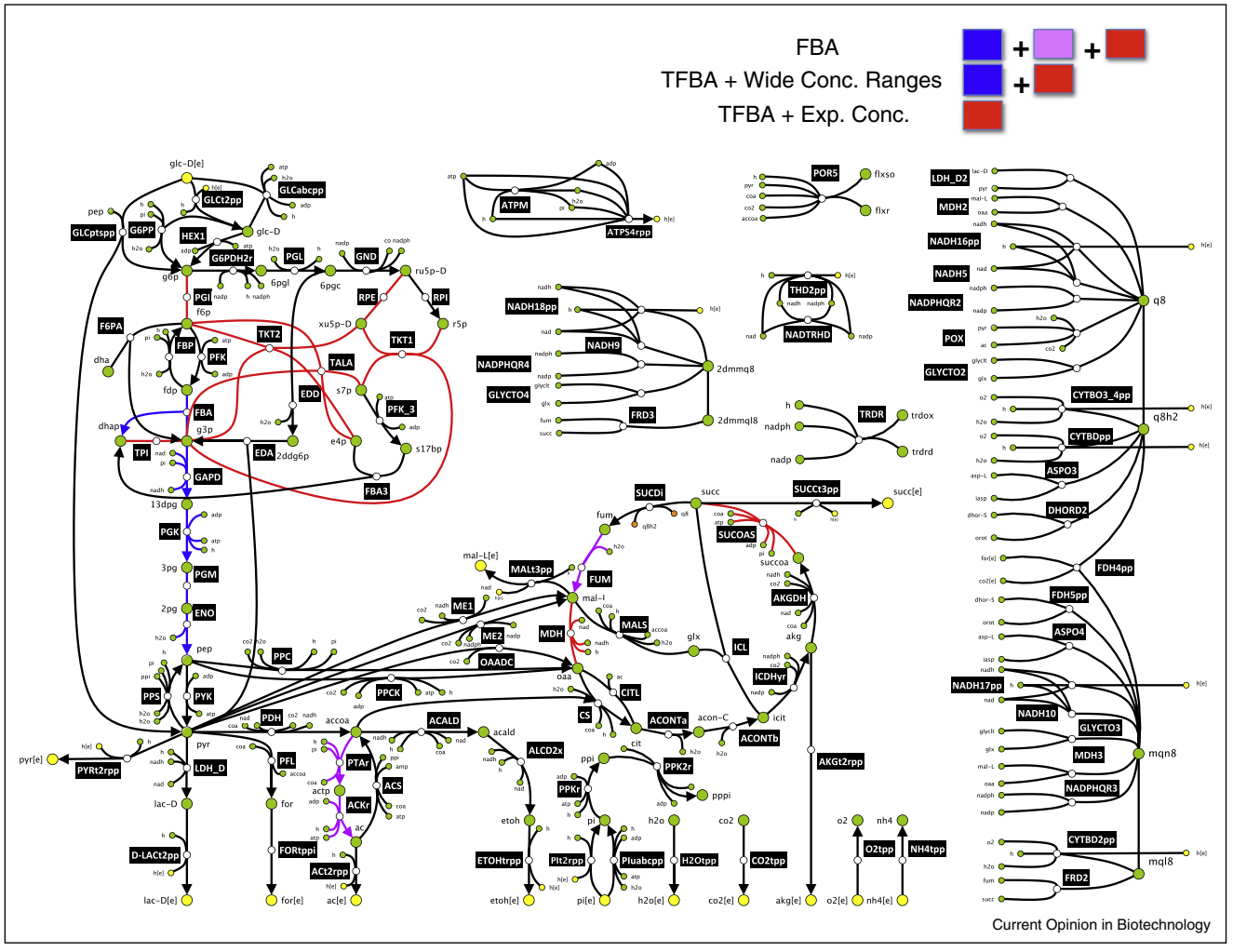
Directionalities of metabolic fluxes in GEMs have significant impact on network properties, such as yield on biomass, gene/reaction essentiality. Directionality specifications in GEMs are based on literature, databases, biochemical textbooks and information from similar

Figure 1



Applications of thermodynamics in metabolic networks. Standard Gibbs free energy values can be used to evaluate pathways, and to assign directionalities in GEMs. Integration of network-thermodynamics allows computation of thermodynamically feasible flux profiles and EFMs and feasible concentration ranges. These ranges can be further used for estimating the equilibrium displacements and building kinetic models of metabolic networks.

Figure 2



Bidirectional reactions in reduced *E. coli* network. Without any thermodynamic constraints, the reactions colored with blue, red and purple are assigned as bidirectional by FBA. By applying TFA on the network with wide metabolite concentration ranges, the reactions colored with blue and red are bidirectional. Integration of metabolomics data [51] to TFA constrains 3 more reaction as unidirectional (colored with red). Using only standard Gibbs reaction values for directionality assignment cannot capture the impact of concentration ranges on directionality of reactions.

organisms. If there is no available information about the catalytic reversibility of an enzyme, the corresponding metabolic reaction is defined as *bidirectional* in the network. The sole systematic approach then to account for the directionality for these reactions is integration of thermodynamics into metabolic networks. In a recent study, Dreyfuss *et al.* [9] reconstructed the genome scale metabolic model of *Neurospora crassa*, and by using the Gibbs free energy of reactions through the Group Contribution Method (GCM) [10], they constrained 1046 of 1374 metabolic reactions as unidirectional. However, they did not take into account the contribution of the activities to the Gibbs free energy of reactions, and they determined the directionality just by using the standard values. Pitkanen *et al.* [11] followed the same path, and they assigned directionalities to the reactions in 49 fungal species' GEMs by using eQuilibrator [12]. Assigning directionality based on the Gibbs free energy approach is becoming more and more popular, and has been applied to many metabolic network reconstructions [13–19]. All these calculations are done without a systematic integration of thermodynamics constraints. A systematic approach provides a more accurate estimation on the directionality of reactions in metabolic networks rather than using only the standard Gibbs free energy of reactions (Figure 2).

Thermodynamic constraints can also be used to test the consistency of pre-determined directionality in GEMs. By utilizing the NET analysis method, Martinez *et al.* [20•], identified 319 unidirectional reactions in Recon 1, human metabolic network model. 306 of these reactions were already set as unidirectional in GEM, whereas 13 of them emerged as new unidirectionality

constraints. They also revealed thermodynamically infeasible internal loops, and removed these biologically meaningless futile cycles. Moreover, they concluded that organisms use alternative methods in order to overcome the thermodynamic constraints, such as substrate channeling or coupling with ATP hydrolysis. In this study as in all the current studies that use GCM data, the standard Gibbs free energy values used were estimated for 25 °C, while the human body temperature is typically 12 degrees higher. This points to one of the important needs for expanding the GCM methods for broader ranges of temperature and pressure that can be used for the analysis of systems like human and extremophiles.

Improving model predictions by integrating thermodynamics with metabolic models

Accurate prediction of the observed phenotypes is one of the main evaluation criteria of the quality of metabolic models. Thermodynamics, along with other constraints, such as mRNA and protein expression data, are widely used to improve the prediction capacity of metabolic networks. For instance, by applying thermodynamics constraint in a systematic manner by TFA, Soh *et al.* [21] showed that the optimum specific growth rate of *Saccharomyces cerevisiae* drops from 1/h to 0.42/h with 15.33 mmol/gDWhr glucose uptake rate, and the experimentally observed value was 0.35/h. In another study, Schulz and Qutub proposed a method (corsoFBA) to integrate a 'thermodynamics cost' to metabolic networks, similar to EBA analysis, while optimizing the protein cost to study sub-optimal growth phenotypes. They succeeded in capturing different metabolic states at sub-optimal growths from experimental data of *Escherichia coli* at different dilution rates [22].

NET analysis has been also used to test the thermodynamic feasibility of given directionalities in metabolic networks. De Martino and colleagues [23] proposed a method that attempts to overcome the NET analysis' requirement of pre-assigned reaction directionalities. They used their method to estimate the metabolite concentration ranges for human red blood cells and to identify thermodynamically infeasible loops in *E. coli*. However, their formulation cannot guarantee a globally optimal solution and cannot enumerate alternative optimal solutions as it is done by the TFA.

In another study, McCloskey *et al.* combined NET and experimental analysis on *E. coli* [24••]. The authors revealed that the Acetyl-CoA C-acetyltransferase is thermodynamically infeasible in acetoacetylCoA synthesis direction. Under anaerobic and aerobic conditions, this infeasibility results in a 2.9% and 1.1% reduction in growth, respectively.

Orman *et al.* [25•] used EBA analysis to study the behavior of the perfused livers under fed and fasted states. They applied EBA on certain parts of the network, such as pathways, and assumed that the overall dissipation of these pathways must be equal or greater than 0. The result was a significantly reduced solution space.

These studies further demonstrate the benefit of thermodynamic constraints and it will be interesting to compare their results with the TFA analysis, which is not subject to preassigned reaction directionalities.

Thermodynamics methods in systems and synthetic biology tools

Integrating thermodynamic information, either through Gibbs free energy of formation, or in a network manner such as TFA, has become one of the most important features of systems biology and metabolic engineering for the analysis and design of synthetic metabolic pathways.

Pathway design tools [26–30] use the standard Gibbs free energy of reactions estimated through GCM [10] to prune the set of *de novo* generated pathways and to retain only the thermodynamically feasible ones. However, they do not include any systematic network-thermodynamics approach to account for the effect of concentrations of metabolites on the overall thermodynamic feasibility of the pathways. The necessity of adjusting the estimated standard Gibbs free energies to physiological conditions (metabolite concentrations, pH, and ionic strength) is discussed in [31,32]. These studies also demonstrate that the number of feasible pathways can be reduced significantly if thermodynamic constraints are applied. The pruning that is based only on the standard Gibbs free energy, and using methods like EBA, is very conservative. Many pathways that would be discarded as infeasible using standard Gibbs free energy could become feasible using a framework like TFA, which allows for adjustment of the free energy to physiological conditions.

The use of thermodynamics in systems biology tools is not limited to pathway evaluations and can also be used to provide ranges for the flux values. Thermodynamic Optimum Searching (TOS) [33] aims to calculate the thermodynamically optimal flux solution by minimizing the magnitude of Gibbs free energy change and maximizing the entropy production with an EBA type analysis. Muller and Buckmayr [34] propose a similar method with improved computation time. However, these methods are based on the formulation of EBA, and therefore their results are biased in the pre-selection of Gibbs free energy bounds. It remains to be shown how the reformulation of these methods can integrate metabolite concentrations as variables and how they can reduce the computational cost associated with such integration.

Thermodynamically feasible elementary flux modes

Elementary Flux Modes (EFMs) analysis, which characterizes the allowable steady state fluxes for a metabolic

network [35], has been extensively used to investigate the capabilities of metabolic networks. However, even a small network can have millions of EFMs, and this necessitates the usage of methods to characterize and eliminate biologically irrelevant EFMs. Gerstl *et al.* [36•] developed a framework to identify thermodynamically feasible EFMs by utilizing NET approach. This method reduced the number of EFMs significantly by eliminating the thermodynamically infeasible EFMs. A very similar approach was followed by Jol *et al.* [37], in which they calculated 71 million EFMs of *S. cerevisiae* metabolic network, and through the NET analysis, they concluded that 56% of the EFMs are thermodynamically feasible. A method that integrates thermodynamics into EFM analysis with improved efficiency has been also developed [38]. These are very promising results and new model formulations and algorithms can lead to significant reductions in the number of EFMs, removing one of the main limitations for their broader applicability.

Thermodynamically feasible concentration ranges, kinetic modeling and metabolomics

We can further use network thermodynamics to integrate metabolomics data in metabolic network models and to evaluate the consistency of these data with the metabolic flux profiles. Since metabolite concentrations determine the Gibbs free energy of a reaction, the concentrations of the reactants must be consistent with the flux directionality. Network thermodynamics can also be used to validate the experimental results by *in silico* predictions, such as measured metabolite concentrations [39], or to explain the variations in concentration levels under different growth conditions [40].

Soh and Hatzimanikatis [4] defined the thermodynamic space of the network as the space of the thermodynamically allowable metabolite concentrations and the space of the reaction displacements from equilibrium, which are constrained by the Gibbs free of the reactions in the network. The thermodynamic space of a metabolic network can be characterized and analyzed through the sampling of the concentrations of the metabolites and/or the corresponding Gibbs energies of reactions using a TFA or a NET analysis formulation. Using such approaches Soh *et al.* [21] have derived for the first time the displacement from equilibrium for a metabolic network in yeast, which includes the central carbon pathways and the cytosolic and mitochondrial electron transport chains. A similar approach was also used by Birkenmeier *et al.* [41] to generate thermodynamically feasible pathways, by sampling the metabolite concentrations by NET analysis approach. They analyzed the glycerol biosynthetic pathway of *S. cerevisiae* without a detailed knowledge of enzyme kinetics. They concluded that the pathway is primarily controlled by glycerol-3-phosphate dehydrogenase enzyme that operates far from equilibrium; which was previously proven experimentally.

Thermodynamically feasible steady state concentration profiles can be further used for kinetic analysis of organisms. Chakrabarti *et al.* [42••] developed a method to build kinetic models for genome scale reconstructions that takes into account all the stoichiometric and thermodynamic constraints of the flux balance models. In this work, they calculated the thermodynamic space in a metabolic network of the 146 reactions and 90 metabolites that describe the central carbon metabolism and electron transport in *E. coli* using the TFA formulation. For each sampled set of the thermodynamic allowable concentrations, they next calculated thousands of kinetic models that were stable and consistent with the allowable concentrations and flux profiles. The efficiency of the method in building kinetic models of such size and quality depends strongly on the proper choice of thermodynamically feasible concentrations early in the model-building process. Similar studies by Milo and colleagues [43,44] have shown that the integration of thermodynamic constraints and the decomposition of the rate expressions between the kinetic and the thermodynamic terms can improve the process of building kinetic models and provide important insights into the analysis of complex kinetic models.

Conclusions

Although thermodynamics have been used in many studies, their use is still limited relative to the enormous field of metabolic modeling. Plants, due to their importance for energy capture, and extremophiles, due to their non-standard bioenergetics properties, are very promising organisms for biotechnology but the study of their bioenergetics is very challenging. Simons *et al.* [45] reconstructed a metabolic model of a maize leaf and they used the Gibbs free energy of reactions to remove thermodynamically infeasible cycles. PlantSEED [46•], a comprehensive computational environment that focuses on plant metabolism, includes in its database thermodynamic properties of the metabolites and reactions, and it defines cellular subsystems and compartments based on metabolic reconstructions for plants. Introduction of thermodynamic constraints in metabolic models that include multiple compartments is a challenging task that will require careful formulation of the thermodynamic constraints for the transport reactions [47]. TFA formulations for such networks can reveal important properties of the energy metabolism and the bioenergetics properties of plants and other multicompartmental organisms.

A recent study [48] on the adaptation of *Saccharomyces* species to different temperatures, accounted for the Gibbs free energies of reactions under different conditions, and predicted the metabolic changes that keep cells alive, such as increased glycerol accumulation. To perform detailed study on organisms under such harsh conditions, it is essential to have accurate predictions for

thermodynamics properties at high and low temperatures or high pressures. Recently, with the integration of quantum chemistry, this issue has been addressed, and new methods are proposed to achieve this goal [49•,50].

Regardless of the organism under study, all future studies will require development of methods for: (i) the identification and reduction of number of bidirectional reactions, and (ii) the identification of the metabolites which, if measured, would allow us to estimate the displacement of reactions from thermodynamic equilibrium with higher confidence. Using methods like EBA and TFA we can identify the number of the bidirectional reactions. However, alternative combinations of flux directionalities can grow enormously as the number bidirectional reactions increases. Methods that can rank the alternative flux directionalities and the associated flux profiles can provide a systematic analysis of cellular physiology. Similarly the identification of the most informative metabolites will be very important for studies in metabolomics, physiology and bioenergetics. These methods should be able to handle large-scale to genome-scale networks and to account for the uncertainty in the input data. We expect that developments in these areas will further expand the scope and the usefulness of network thermodynamics.

Acknowledgments

M.A. and V.H. were supported by the Ecole Polytechnique Fédérale de Lausanne (EPFL), the Swiss National Science Foundation, and the RTD grants MetaNetX and MicroscapesX within SystemsX.ch, the Swiss Initiative for Systems Biology evaluated by the Swiss National Science Foundation.

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